

Conserved selenoprotein synthesis is not critical for oxidative stress defence and the lifespan of *Drosophila*

Mitsuko Hirosawa-Takamori, Ho-Ryun Chung & Herbert Jäckle⁺

Max-Planck-Institut für Biophysikalische Chemie, Abteilung Molekulare Entwicklungsbiologie, Am Fassberg, Göttingen, Germany

Selenoprotein synthesis is conserved from bacteria to man. It involves the differential decoding of the UGA stop codon as selenocysteine. The proteomes of both prokaryotes and eukaryotes, with the exception of yeast, contain only few selenoproteins. This low number is explained by a counterselection of readily oxidized selenocysteine after the introduction of oxygen into the atmosphere and the need to conserve selenoenzymes that control redox homeostasis of cells. Lack of selenoprotein synthesis in vertebrates impairs the oxidative stress defence and causes lethality. Here we show that *Drosophila* mutants that lack the translation elongation factor SelB/eEFsec fail to decode the UGA codon as selenocysteine, but they are viable and fertile. Oxidative stress responses and the lifespan of these flies are not affected. Protecting cells from oxidative stress can therefore not account for the selection pressure that conserves selenoprotein biosynthesis during the course of evolution.

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INTRODUCTION

Selenoproteins are implicated in oxidative stress defence and in a number of clinical syndromes including cancer in man (Moustafa *et al*, 2003). Their synthesis depends on the differential decoding of the UGA stop codon as selenocysteine (Atkins & Gesteland, 2000; Böck, 2000). In bacterial mRNA, this process is regulated by a special 'stem-loop' structure, termed the SECIS element, next to the UGA codon. This element binds SelB, an EF-Tu-like elongation factor, which associates with selenocysteine-tRNA. Functional formation of the SECIS–SelB–tRNA complex ensures that the neighbouring UGA is read as selenocysteine instead of a stop codon (Böck, 2000). In higher organisms such as archaea, flies and man, the selenocysteine-coding machinery is conserved but modified. First, the SECIS element is located far from the UGA codon in the 3' untranslated region of the mRNA (Low & Berry,

1996). Second, the SelB function is provided by two proteins, termed eEFsec and SECIS-binding protein 2 (SBP2) (Copeland *et al*, 2000; Fagegaltier *et al*, 2000; Tujebajeva *et al*, 2000). The available evidence therefore suggests that selenoprotein synthesis predates the diversion of bacteria, archaea and eukaryotes, and that the principle of synthesis has been conserved during the course of eukaryotic evolution (Low & Berry, 1996; Atkins & Gesteland, 2000; Hirosawa-Takamori *et al*, 2000; Driscoll & Copeland, 2003).

In mammals, a number of key enzymes involved in oxidative stress defence carry a UGA-coded selenocysteine in their enzymatic centres (Driscoll & Copeland, 2003). As both vertebrates and invertebrates have highly efficient oxidative stress defence systems (Melov, 2002), the need for selenoproteins for the control of redox homeostasis appears to be the likely selective force by which selenoprotein synthesis has been conserved throughout the animal kingdom. Here we report that the *Drosophila* translation elongation factor SelB/eEFsec is required for the differential decoding of the UGA codon. SelB/eEFsec mutations impair the differential UGA-decoding mechanism, but viability, lifespan and oxidative stress reactions of the mutants are not affected. This surprising finding challenges the view that a selenocysteine-based oxidative stress defence system is the key in conserving the selenoprotein biosynthesis system during the course of evolution.

RESULTS AND DISCUSSION

In *Drosophila*, a genetically amenable invertebrate, three selenoprotein-coding genes were previously identified by homology cloning and whole-genome sequence analysis (Hirosawa-Takamori *et al*, 2000; Castellano *et al*, 2001; Martin-Romero *et al*, 2001; Kryukov *et al*, 2003). To assess the maximal effect of loss of selenoprotein biosynthesis in flies, we searched for the SelB/eEFsec homologue by *in silico* analysis of the genome, cloned the gene and generated a SelB/eEFsec knockout mutation.

Structure and expression of *Drosophila* SelB/eEFsec gene

The structure of the *Drosophila* SelB/eEFsec gene, as revealed by sequence comparison of cDNA and corresponding genomic DNA, is shown in Fig 1A. It is located on the right arm of the second chromosome in section 57E and contains two exons that code for a single transcript of ~1.6 kb (Fig 1A). Comparison of SelB/eEFsec

Max-Planck-Institut für Biophysikalische Chemie, Abteilung Molekulare Entwicklungsbiologie, Am Fassberg, D-37077 Göttingen, Germany

⁺Corresponding author. Tel: +49 551 2011482; Fax: +49 551 2011755;

E-mail: hjaeckl@gwdg.de

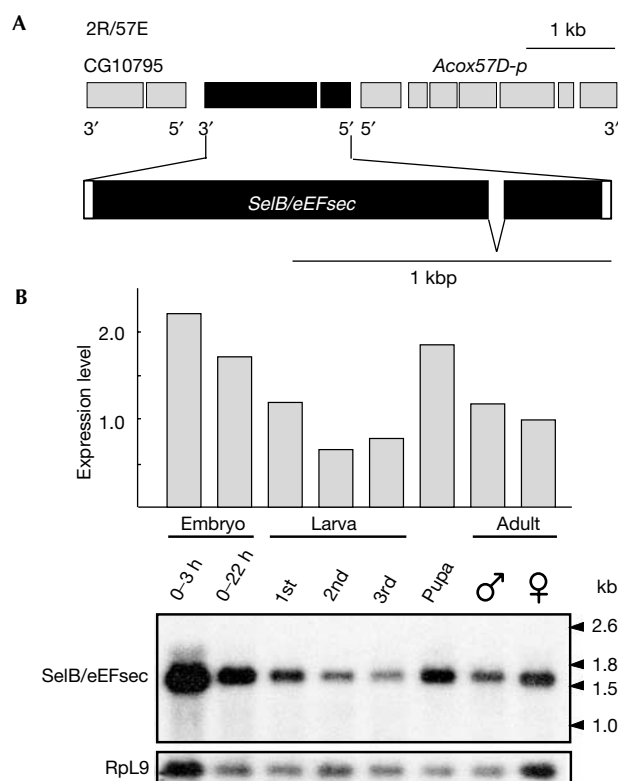


Fig 1 | Structure and expression of *Drosophila* SelB/eEFsec. (A) Genomic organization of *Drosophila* SelB/eEFsec showing that the gene is composed of two exons; coding sequences (black bars) and untranslated regions (open bars) are indicated. (B) Developmental expression of SelB/eEFsec transcripts as revealed by northern blot analysis using a SelB/eEFsec-specific probe; the amount of SelB/eEFsec mRNA was normalized against RpL9 transcripts serving as an internal control.

of *Drosophila* and mouse (Fagegaltier et al, 2000; Tujebajeva et al, 2000) revealed 40% similarity. SelB/eEFsec mRNA could be detected during all stages of the *Drosophila* life cycle, with high levels during early embryogenesis, a decrease during larval stages and enrichment in pupae and adults (Fig 1B).

Generation of SelB/eEFsec mutant alleles

SelB/eEFsec knockout mutations were generated by homologous recombination (Rong & Golic, 2000; Rong et al, 2002). Briefly, we generated transgenic flies containing a nonfunctional SelB/eEFsec gene having the two TAA stop codons and a frameshift in the coding region (Fig 2A,C). We screened 1,000 individuals for a recombination event (Fig 2A) and identified two SelB/eEFsec mutants (KO#24 and KO#46) (Fig 2B) that carry deletions of ≤ 1 kb and carry both TAA stop codons as well as the altered reading frame (Fig 2C; see Material and methods). RT-PCR showed that only mutant mRNA is expressed in the mutants (Fig 2D). Furthermore, antibodies directed against a C-terminal polypeptide detect SelB/eEFsec on western blots of protein extracts derived from both wild-type flies and flies that over-express SelB/eEFsec from a cDNA-based transgene, but not in extracts from homozygous SelB/eEFsec individuals (Fig 2E). This result indicates that the SelB/eEFsec knockout mutants, which

carry a deficiency corresponding to the essential N-terminal region of the protein, are null mutations.

SelB/eEFsec mutants lack differential decoding activity

Mouse mutants that are unable to synthesize selenoproteins do not survive (Bosl et al, 1997; Kumaraswamy et al, 2003). In contrast, SelB/eEFsec mutants of the genotypes KO#24/KO#24, KO#46/KO#46 or KO#46/Df(2R)PK1 develop into fertile flies, indicating that the gene has no vital function.

We next examined whether SelB/eEFsec mutants are able to decode differentially the UGA codon. We generated transgenic flies carrying the previously described LacZ/UGA/luciferase reporter genes either with or without a SECIS element (Hirosewa-Takamori et al, 2000). Wild-type transformants containing the SECIS-bearing reporter gene contained luciferase activity, whereas homozygous SelB/eEFsec mutants contained only background levels both in the absence and presence of the SECIS element (Fig 3A). The ability to produce SECIS-dependent readthrough activity was rescued by the expression of one copy of SelB/eEFsec transgene under control of the actin 5C promoter (see Hirosewa-Takamori et al, 2000) in the mutant flies (Fig 3A). Furthermore, antibodies directed against *Drosophila* selenoprotein SPS2 (Hirosewa-Takamori et al, 2000; Castellano et al, 2001; Martin-Romero et al, 2001) detect the selenoprotein on western blots containing wild-type protein extracts, whereas no SPS2 was found in SelB/eEFsec mutants (Fig 3B). Collectively, these results establish that flies lacking SelB/eEFsec activity are unable to decode UGA as selenocysteine.

No effect on longevity and oxidative stress defence

Homozygous SelB/eEFsec mutant individuals develop into fertile adults, which can be propagated in a wild-type-like manner. This finding shows that the mutation has no immediate effect on viability. Furthermore, the lifespan of the mutant individuals (Fig 4A) and their response to oxidative stress (Fig 4B,C) was indistinguishable from wild type.

The finding that selenoproteins are not essential for redox homeostasis of *Drosophila* is consistent with the observation that *Drosophila* and *Anopheles* homologues (H.-R. Chung, unpublished observation) of vertebrate antioxidant enzymes such as glutathione peroxidase, thioredoxin reductase or SelR are non-selenocysteine (Kryukov et al, 2002; Missirlis et al, 2002, 2003). However, it seems to contradict recent results showing that suppression of *Drosophila* SelH (BthD) by RNAi expression reduces the viability of flies (Kwon et al, 2003). In this context, it is important to note that the *Drosophila* genes encoding SelH, SelD and SelK are duplicated and code for non-selenoproteins, which contain cysteine or arginine instead of the selenocysteine residue (Adams et al, 2000; Castellano et al, 2001; Martin-Romero et al, 2001). In the case of the two SelD homologues (SPS1 and SPS2), the non-selenoprotein SPS1 was shown to exert a vital function and participates in the oxidative stress defence (Morey et al, 2003a,b). Thus, its activity may provide a backup function to support selenoprotein SPS2 activity in the SelB/eEFsec mutant individuals. By analogy, the non-selenoprotein homologue of SelH might compensate for the loss of selenoprotein SelH function in SelB/eEFsec mutant individuals, whereas in RNAi knockdown mutants the activities of both SelH proteins might be impaired on the basis of their similar mRNA sequences. This may therefore

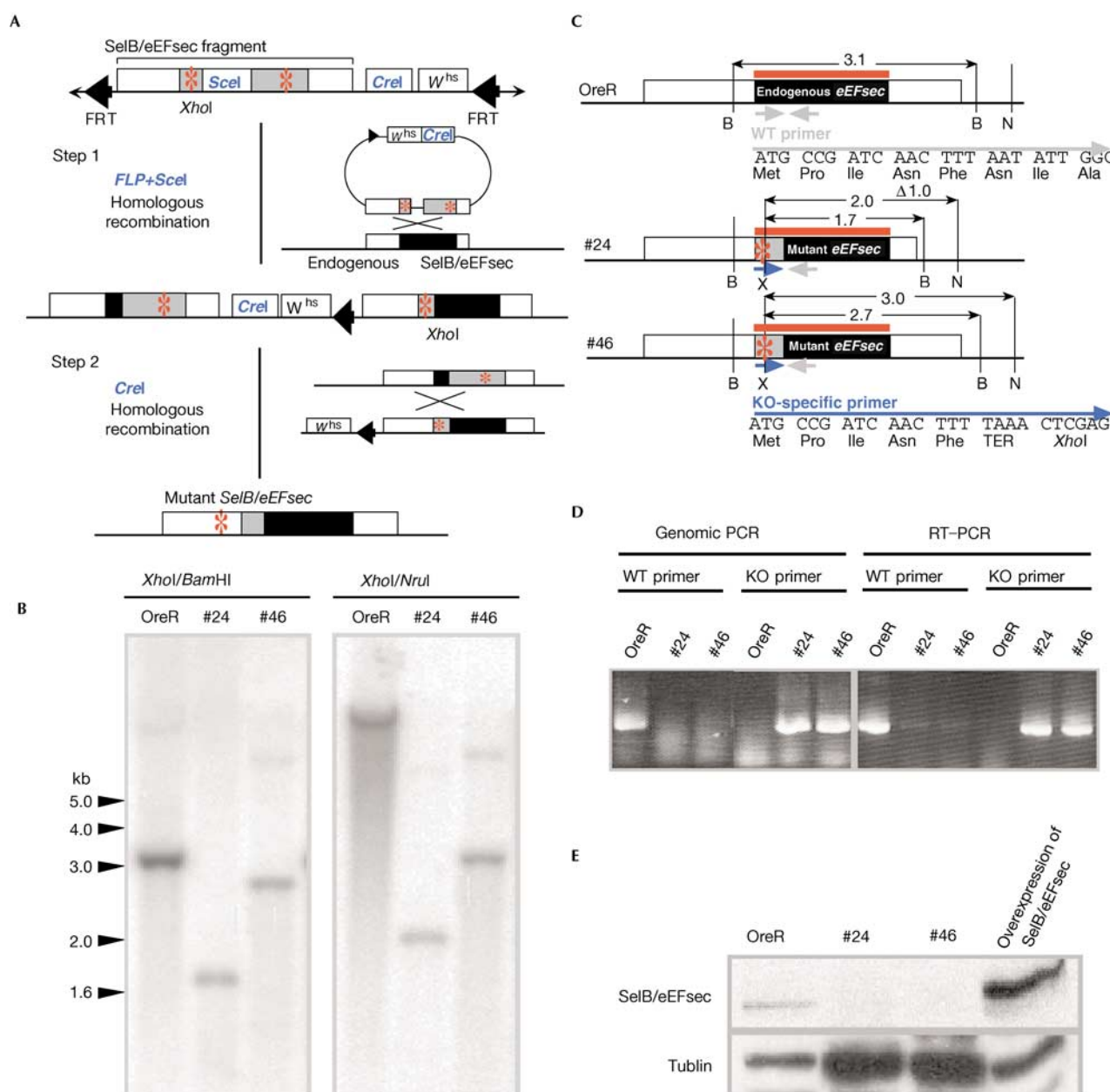


Fig 2 | Schematic representation of SelB/eEFsec mutagenesis by homologous recombination. (A) The targeting DNA fragment excised by FLP from the donor construct is expected to recombine with endogenous SelB/eEFsec locus (step 1; for details, see Rong & Golic, 2000; Rong et al, 2002). In a second step, the tandem duplication was reduced to a single copy by a homologous recombination event due to a CreI-mediated double-strand break (step 2). Red asterisks: introduced TAA stop codon. (B) Southern blot with genomic DNA from wild-type and homozygous knockout mutant (KO#24 and KO#46) individuals. DNA was digested by XhoI, which is the newly generated site only in mutant DNA (see (A)), and by either BamHI or NruI. The SelB/eEFsec-specific DNA hybridization probe is indicated by red lines. (C) Gene structure of the wild-type (shown on top) and of SelB/eEFsec mutant alleles (#24 and #46). Restriction sites are as follows: B (BamHI), N (NruI) and X (XhoI). Size of DNA fragments in kilobase pairs (kb). (D) Genomic DNA and total RNA were prepared from wild-type (OreR) or homozygous knockout mutant flies (KO#24 and KO#46). Wild-type and knockout-specific primers are shown in (C); for their sequence, see Material and methods. (E) Anti-SelB/eEFsec antibody staining of western blots loaded with protein extracts of wild-type flies (OreR), homozygous knockout mutant flies (KO#24 and KO#46) or flies overexpressing SelB/eEFsec from a cDNA-derived transgene (see text). Note that SelB/eEFsec is left undetected in the KO#24 and KO#46 mutant individuals even in the presence of higher amounts of loaded protein (see loading control provided by anti- α -tubulin staining).

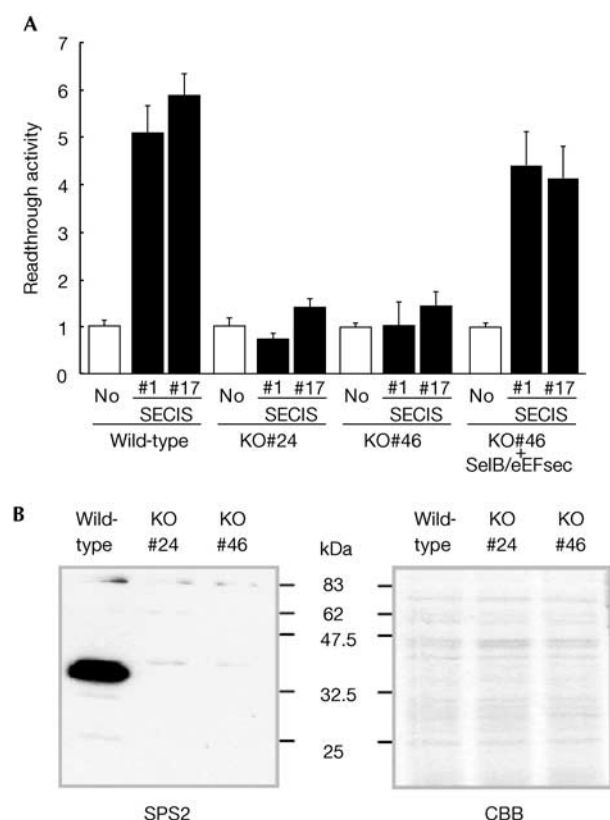


Fig 3 | Lack of readthrough activity in SelB/eEFsec knockout mutants carrying a LacZ/UGA/luciferase reporter gene. (A) LacZ/UGA/luciferase reporter gene activity of transgenes lacking (No) or containing the SECIS element (#1 and #17 represent different transgenic lines) in wild type, in two different *SelB/eEFsec* mutants (KO#24 and KO#46) and *SelB/eEFsec* mutants that contain a cDNA-based *SelB/eEFsec*-expressing transgene (KO#46 + SelB/eEFsec). For details of the assay system, see Hirosawa-Takamori *et al* (2000). Bars represent the mean values of relative luciferase activities from six independent experiments; standard deviation is indicated. Note SECIS-dependent readthrough activity in wild type, lack of readthrough activity in *SelB/eEFsec* mutants and the *SelB/eEFsec* transgene-dependent rescue of the readthrough activity. (B) Anti-dSPS2 antibody staining of western blots containing protein extracts of wild-type (white) and homozygous knockout mutant (KO#24 and KO#46) flies. Note the absence of SPS2 protein in the *SelB/eEFsec* mutant individuals. CBB, Coomassie brilliant blue stained gel.

explain the different effects of RNAi knockdown experiments (Kwon *et al*, 2003) versus the selective loss of selenoproteins in SelB/eEFsec mutants described here.

SPECULATION

Our results present evidence that *Drosophila* selenoproteins are not essential for viability, longevity or oxidative stress defence as observed with vertebrates (Low & Berry, 1996; Driscoll & Copeland, 2003). Thus, selenoproteins may have undergone an insect-specific adoption of novel function(s) once the components of their oxidative stress defence system became independent of selenoprotein biosynthesis. This conclusion is consistent with the most recent finding of species-specific selenoproteins in lower

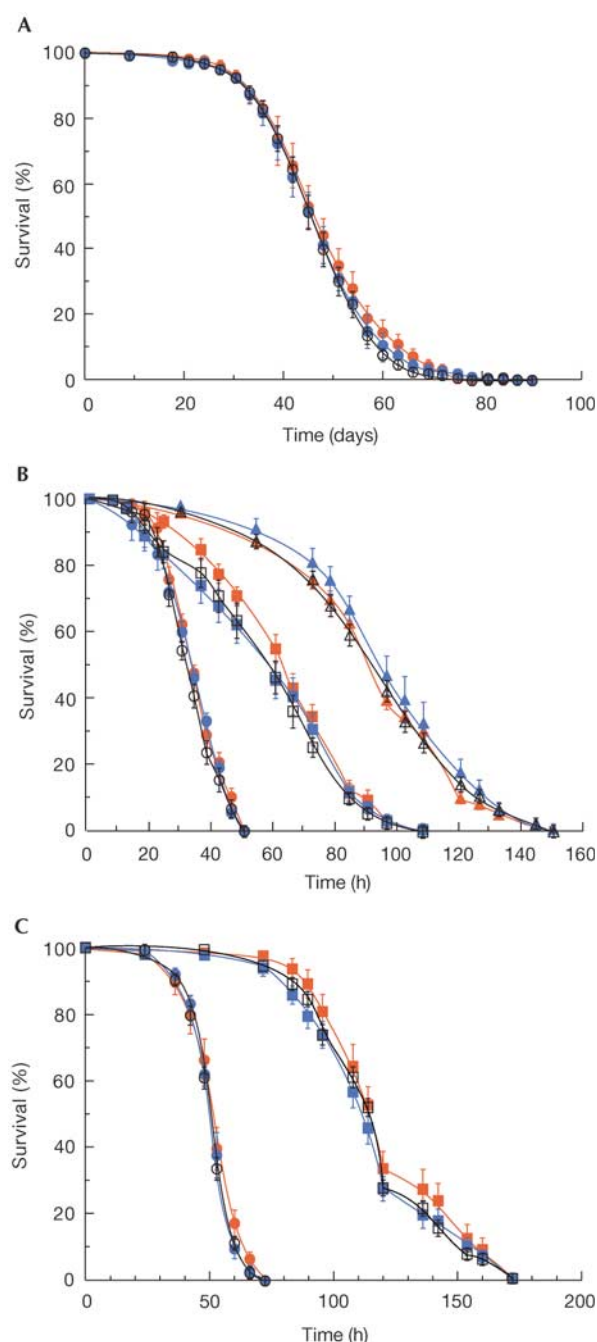


Fig 4 | Determination of lifespan and sensitivity to oxidative stress of SelB/eEFsec mutant flies. (A) Comparison of the lifespan (in days) of wild-type (open circles) and *SelB/eEFsec* mutant (red: KO#24; blue: KO#46) individuals ($n > 150$ males per genotype; mean value of three independent experiments). (B) Survival of wild-type and mutant flies (for coding, see (A)) at different paraquat concentrations (40 mM: circles; 10 mM: squares; 2.5 mM: triangles). (C) Survival of wild-type and mutant flies (for coding, see (A)) at different hydrogen peroxide concentrations (3.0%: circles; 0.3%: squares).

vertebrates, which are not conserved in mammals (Castellano *et al.*, 2004).

The fact that selenoprotein biosynthesis is maintained in flies suggests that following initial gene duplication events in ancestral organisms, selenoprotein-coding genes may have adopted new and possibly important, but nonvital, functions. Such functions may affect, for example, behaviour, learning and/or memory processes, which were not addressed by our present study, and may account for the continued requirement for selenoprotein synthesis once the redox homeostasis system became independent of selenocysteine-bearing enzymes during the course of insect evolution.

MATERIAL AND METHODS

Fly stocks and genetics. Flies were kept as described (Missirlis *et al.*, 2002). To obtain the donor flies for homologous recombinations, the targeting DNA construct was transformed into flies by P-element transformation (Rubin & Spradling, 1982). Stocks for homologous recombination were *yw* (*v*); *P{ry⁺,70FLP}4*, *P{v⁺,70I-Scel}2B*, *Sco/S²*, *CyO* and *yw*; *P{I-Crel}/TM3*, *Sb*, *Ser*. Targeting was performed as described (Rong & Golic, 2000; Rong *et al.*, 2002). Transgenic flies (No. #1, #17) and the assay used to examine the readthrough activity of the UGA codon are described by Hirosewa-Takamori *et al.* (2000). The DNA fragment containing the SelB/eEFsec open reading frame was cloned into the pUAST vector DNA (pUAST-eEFsec) and used for fly transformation (see above). Crosses were carried out to obtain KO#24 or KO#46 mutant flies bearing the transgene to assay whether selenocysteine coding is restored in mutants. Protein extracts were prepared for each genotype (Fig 3B), and LacZ and luciferase activity were assayed (Hirosewa-Takamori *et al.*, 2000).

Molecular characterization of the target event. Southern blot analysis was performed using the *EcoRI/XhoI* fragment of LP02881 DNA as probe (Fig 2B). Preparation of genomic DNA, total RNA and amplification by genomic PCR have been described (Missirlis *et al.*, 2002). PCR involved wild-type primers (5'-ATGCCGATCAACTTTAATATTGGC-3') and KO-specific primers (5'-ATGCCGATCAACTTTTAACTCGAG-3') and the common reverse primer (5'-GAGCATGAGATCAATGATCTGGGCACCTCC-3'). RT-PCR was carried out as outlined by the manufacturer (Promega, Madison, USA).

Targeting vector construct. Two genomic DNA fragments (fragments 1 and 2) were amplified by genomic PCR. Primers were P1F (5'-GCGGCCGCGCCTTACCTGAGCATGTGCGATC-3') and P2R (5'-GCTAGCCCGCAAGCGTAGCTTCTCCAGCTT-3') for fragment 1; and P3F (5'-AAGCTTCTCGAAAACTTTGGAAGCACC-3') and P4R (5'-GGTACCACAGGCGCTGTGCTGCCTTAACC-3') for fragment 2. To introduce base-pair changes, fragments were cloned into pCRII-TOPO DNA (Invitrogen, Groningen, The Netherlands). Mutagenesis was performed with the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, USA). Primers were 5'-GTACAATGCCGATCAACTTTTAACTCAGTTGCTGGGACATGTTGAC-3' and 5'-GTCAACATGTCCAGCAACTCGAGTTTAAAGTTGATCGGCATTGTAC-3' to introduce TAA stop codons, a *XhoI* site and a frame shift in the wild-type sequence. The primers to generate a *Scel* site were 5'-CTGGCTAGGGATAACAGGGTAATA-3' and 5'-AGCTTATTAACCTGTTATCCCTAG-3'. Mutated fragment 1 DNA was generated by *NotI/NheI* treatment, and mutated fragment 2 DNA by *HindIII/*

KpnI digestion. The fragments were ligated into the *NotI/KpnI* sites of pTV2 vector DNA (Rong *et al.*, 2002) using the linker *Scel* oligos.

Developmental expression analysis and western blot analysis. Developmental northern blot analysis was performed as described (Grönke *et al.*, 2003). Radioactively labelled SelB/eEFsec antisense RNA probe was prepared by *in vitro* transcription on an *EcoRI*-linearized LP02881 cDNA template using SP6 polymerase (Strip-EZ RNA kit; Ambion, Austin, TX, USA). As loading control, the blot was reprobated with Rpl9 antisense RNA (Grönke *et al.*, 2003). Anti-SelB/eEFsec-specific rabbit antiserum (Eurogentec, Seraing, Belgium) was produced against the amino-acid stretch GEK GRIERTFGQTSK (positions 458–472). Western blots prepared from protein extracts of females were stained with anti-SelB/eEFsec rabbit serum (1:2,000 dilution) or anti-SPS2 rabbit serum (1:1,000 dilution). As secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:10,000 dilution; Sigma-Aldrich, Steinheim, Germany) was used and visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA). As loading control, the blot was reprobated with antitubulin antibodies (E7; 1:5,000 dilution; DSHB, Iowa City, IA, USA) and HRP-conjugated anti-mouse IgG (secondary antibodies; 1:10,000 dilution; Sigma-Aldrich, Steinheim, Germany).

Paraquat and hydrogen peroxide assays and lifespan measurements. Up to 15 adult males (3- to 4-day-old) were kept in vials with 1.5 ml medium (1% sucrose, 1.3% low melting agarose) and specified concentrations of paraquat or hydrogen peroxide (Sigma-Aldrich, Steinheim, Germany). Survival and lifespan measurements were described earlier (Missirlis *et al.*, 2002).

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